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NOTE

An improved method for determining relative ^{15}N abundance in ammonium regeneration studies by direct diffusion

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ABSTRACT: Ammonium in micromole quantities may be concentrated directly, with a 65% yield, from 250 ml seawater into 10 µl 0.25 *N* sulphuric acid by diffusion for 6 h if the samples are heated and agitated. This makes it possible to measure ammonium regeneration in water containing heterotrophic microplankton by means of ^{15}N with a minimum expenditure of time, labour, and hazardous chemicals, and a greatly diminished risk of contamination. A recommended diffusion procedure is described, together with tests for reproducibility, recovery, and interference. Results of trials with a mixed alga-rotifer culture and a marine plankton sample are described.

Ammonium is an important nitrogen source for phytoplankton growth (Glibert 1988, Paasche 1988). In general, ammonium is made available to phytoplankton by regeneration by heterotrophic organisms, microheterotrophs (organisms < 200 µm in size) frequently being responsible for most of this activity (McCarthy & Carpenter 1983, Glibert 1988). Regeneration by microheterotrophs is usually measured by isotope dilution (Blackburn 1979, Caperon et al. 1979, Glibert 1988). The dilution of added ^{15}N ammonium by unlabelled ammonium is used together with net changes in the total amount of dissolved ammonium pool to calculate the regeneration rates.

Before the ammonium can be subjected to isotope analysis, it must be stripped from the water and concentrated into a very small sample. This may be achieved in several ways. Distillation followed by evaporation (Glibert et al. 1982) or by microdiffusion (Paasche & Kristiansen 1982) is extremely time-consuming. More recent methods, in which ammonium is precipitated as a mercury salt (Fisher & Morrissey 1985) or extracted into an organic phase in the form of indophenol blue (Dudek et al. 1986, Selmer & Sörensson 1986), may be somewhat less cumbersome but necessitate the use of hazardous chemicals. All these procedures are prone to contamination due to extensive handling of the samples or for other reasons.

Blackburn (1979) used a diffusion technique in his experiments with ammonium-rich sediments. As originally described, the method is useful only with high ammonium concentrations in small volumes. If diffusion is to be applied directly to plankton samples, without prior distillation, it must be scaled up to permit ammonium to be collected from much lower concentrations in much larger volumes of water. This was attempted by Probyn (1987) who found, however, that recovery was insufficient unless diffusion was prolonged for up to 2 wk.

In the direct diffusion method described below, designed to be used in coastal and oceanic water, a combination of heating and shaking is employed. This reduces the time needed for a satisfactory yield to 6 h, whereby a number of samples can be processed within reasonable time with a minimum of handling or noxious chemicals.

Description of the direct diffusion method. Based on a series of trials, we recommend the following procedure. The diffusion is carried out in new 500 ml polyethylene bottles that have been washed with 10% HCl and rinsed with deionized water. Before use, the inner surfaces of each bottle are wetted with 10^{-4} *M* NaOH, and then by a portion of the seawater sample. The bottle is then filled with 250 ml of the sample to be analysed. The total ammonium concentration in this sample, i.e. the sum of NH_4^+ initially present and that added as $^{15}NH_4^+$ and carrier, should be 4 µ*M* (i.e. 1 µmol NH_4^+-N per bottle). Each bottle receives 4 ml 50% NaOH (w/v) and is then quickly closed with a silicone stopper. A 40 mm needle of stainless steel projecting downwards from the stopper into the bottle carries (by means of a 'clamp' made of silicone rubber tubing) a 20×5 mm strip of glass fibre filter (precombusted at 450 °C for 4 h) to which 10 µl 0.25 *N* H_2SO_4 has been applied. The sulphuric acid on the filter must be the only acidic surface within the enclosed space. The

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bottles are placed upright in a temperature-controlled water bath at 40 °C, provided with shaking at 90 strokes min^{-1} with an amplitude of 4 to 5 cm. After 6 h, during which time ammonia diffuses from the alkaline samples into the acid on the filter strips, the latter are removed from the bottles and are dried at 60 to 70 °C for 15 to 30 min while still attached to the needles. Finally the filter strips are transferred to test tubes and dried for another 1 to 2 h. The dried samples may be stored in Vacutainers™ (Becton Dickinson) for at least 4 wk without any significant change in atom % ^{15}N. It is desirable to include some standard samples diffused from deionized water. These are needed to keep a check on contamination by unlabelled nitrogen.

Methods used in the tests. Results reported in this note were obtained following the procedure described above unless otherwise noted.

Ammonium chloride standards of varying ^{15}N content, added from concentrated stock solutions, were used in the diffusion experiments at a final concentration of 4 µ*M*. The atom % ^{15}N was measured by emission spectrometry (Kristiansen & Paasche 1982) using a Jasco Model N-150 N-15 Analyzer. The sample (0.6 to 0.7 µmol NH_4^+-N on the filter strip assuming 65 % recovery) was ground with a glass pestle in a small test tube (both precombusted at 450 °C), together with 0.1 g $CaO\text{-}Al_2O_3$ (1 : 1) mixture (precombusted at 1000 °C) and 0.05 g CuO (precombusted at 700 °C). The ground mixture was sealed into a 5 mm OD Pyrex discharge tube (precombusted at 450 °C) and processed for isotope analysis as described by Kristiansen & Paasche (1982). The isotope ratios were adjusted by means of appropriate standard curves prepared from commercial gas samples of known atom % ^{15}N. The amount of contaminating nitrogen was calculated according to the formula

$$C = P \cdot (A_{std} - A_{diff}) \cdot (A_{diff} - A_{nat})^{-1}$$

where C = contamination in µmol $sample^{-1}$; P = amount of (labelled) nitrogen intentionally added, in µmol N $sample^{-1}$; A_{std} and A_{diff} = atom % ^{15}N in the added nitrogen and in the diffused sample, respectively; and A_{nat} = natural abundance of ^{15}N (0.37 atom % ^{15}N).

Deionized distilled water (DDW) was used throughout in the preparation of standard solutions and blanks. Ammonium concentrations were measured according to Solórzano (1969). The recovery of ammonium in the diffusion experiments was checked chemically after extracting it from the filter strips with DDW. The initial pool of ammonium was measured chemically before isotope addition and diffusion, and the analytical values were used to correct the measured isotope ratios for isotope dilution attributed to this ammonium.

Recovery of total ammonium. The effects of heating and shaking on the chemically measured yield are shown in Table 1. Heating alone, either to 40 °C or to 55 °C, did not produce an acceptable yield. Shaking at room temperature improved the recovery, while the best yield, of about 65 %, was obtained when shaking was combined with heating to 40 °C. By placing the diffusion bottle sideways, thus doubling the surface/volume ratio, the recovery in the absence of shaking was improved from 15 % to 53 %. For practical reasons, 40 °C and shaking as described above were adopted as standard diffusion conditions. At higher temperature and/or increased shaking speed, the filter strips tended to collect moisture, and the silicone stoppers sometimes popped off during diffusion. The resulting ^{15}N-values were erratic or not measurable.

Table 1. Effect of heating and shaking on the recovery, expressed as %, of 1 µmol N in the form of ammonium diffused from seawater. Recovery is given either as mean ± standard error (n = 3) or as mean or single value (n = 2 or n = 1)

Temperature °C	Shaking	No. of samples	Recovery (%)
20	No	3	15.6 ± 1.3
20	Yes	3	36.2 ± 2.0
40	No	2	18.6
40	Yes	3	64.7 ± 2.7
55	No	3	15.2 ± 2.1
55	No[a]	1	52.9

[a] Bottle oriented sideways

Chemical analyses (data not shown) indicated that diffusion for 6 h, with heating and shaking, was required for a recovery of 60 % or better. A time course of isotope recovery, shown in Fig. 1, confirmed that the optimum diffusion time was 6 to 10 h.

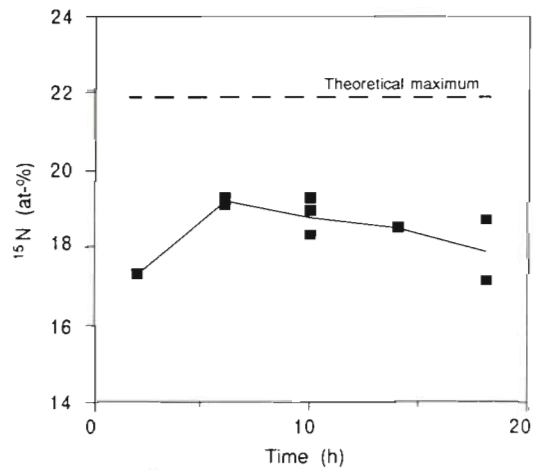

Fig. 1. Atom % ^{15}N in ammonium standards diffused from seawater plotted against diffusion time. The theoretically attainable atom % ^{15}N (horizontal broken line) was corrected for dissolved ammonium (0.18 µ*M*) measured prior to isotope addition

Trials with various bottle types (results not shown) indicate that the diffusion may be carried out in bottles made of polyethylene, polycarbonate or glass. However, as shown in Table 2, there is a risk of a carry-over

Table 2. Content of ^{15}N (atom %) in ammonium standards (1 µmol N per 250 ml sample) of 3 different isotope enrichments, measured either directly or after diffusion from deionized distilled water in new bottles and in bottles previously used at ca 25 at% ^{15}N. Values are given as mean ± standard error (n = 3 or more) or as mean ± deviation from mean (n = 2). (Number of samples, n, in parentheses)

Treatment	Direct (at%)	New bottles (at%)	Used bottles (at%)
Standard 1	0.36 ± 0.01 (n = 3)	0.58 ± 0.04 (n =9)	2.62 ± 0.54 (n = 8)
Standard 2	8.70 ± 0.12 (n = 3)	8.23 ± 0.08 (n = 2)	8.80 ± 0.08 (n = 3)
Standard 3	24.82 ± 0.13 (n = 3)	23.14 ± 0.17 (n = 3)	23.33 ± 0.21 (n = 3)

of ^{15}N if samples with a low atom % ^{15}N are processed in bottles previously used with higher ^{15}N enrichments. This carry-over of ^{15}N was found for all the 3 bottle types tested. The use of new bottles, carefully prepared as outlined above, is recommended for this reason.

Contamination. A calibration curve for ^{15}N ammonium standards diffused from filtered seawater is shown in Fig. 2. The ^{15}N atom % in the diffused samples (corrected for the presence of 0.07 µM NH_4 in the seawater) correlated well (r^2 = 0.998) with that in the

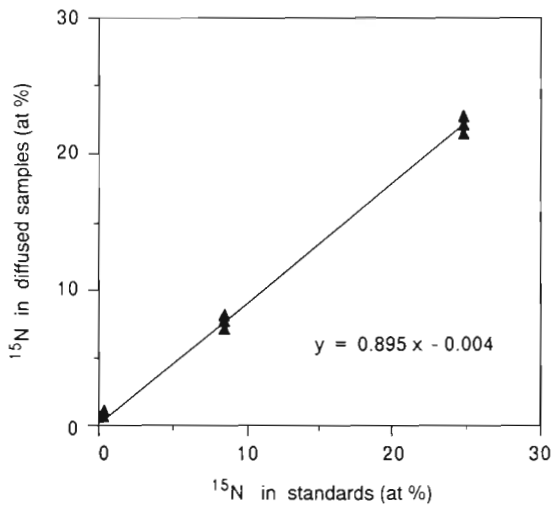

Fig. 2. Atom % ^{15}N in ammonium standards diffused from seawater plotted against the same standards measured directly. The values for diffused samples were corrected for dissolved ammonium (0.07 µM) measured prior to isotope addition. Squared correlation coefficient (r^2) = 0.998

same standard solutions spotted on glass-fibre filter strips and put directly into discharge tubes. The slope of the regression line was < 1, indicating a constant contamination during diffusion; this contamination amounted to 0.12 µmol N per sample. A test involving a comparison also with deionized distilled water (DDW, Table 3) shows that this contamination arose in part (0.06 µmol N sample^{-1}) from general handling of the sample, assuming that DDW is free of ammonium. The

Table 3. Content of ^{15}N (atom %) in ammonium standards (1 µmol N per 250 ml sample) as measured either directly (Direct) or after diffusion from deionized distilled water (DDW), from seawater (SW), or from seawater with an added 1 µmol N per 250 ml of unlabelled (0.37 at% ^{15}N) serine or urea. Contamination is expressed as nmol N 250 ml^{-1} and as % of the NH_4^+–N contained in the sample. Values are means ± standard errors (n = 3) or mean ± deviation from mean (n = 2). The atom % ^{15}N in the seawater was corrected for the presence of 0.07 µM of unlabelled NH_4^+–N

Treatment	No. of samples	Atom % ± SE	Contamination (mean ± SE) nmol sample^{-1}	Contamination (mean ± SE) %
Direct	3	25.19 ± 0.02	–	–
DDW	3	23.77 ± 0.06	61 ± 2	6.1 ± 0.3
SW	3	22.62 ± 0.08	116 ± 4	11.4 ± 0.4
SW + serine	2	21.62 ± 0.14	168 ± 7	16.5 ± 0.7
SW + urea	3	22.96 ± 0.01	99 ± 1	9.7 ± 0.1

rest (0.06 µmol N sample^{-1}; Table 3) would be specifically associated with the seawater.

The filtered seawater used in these tests was shown by one of us (S. Kristiansen unpubl.), using the method of Solórzano & Sharp (1980), to contain 15 to 20 µM N of dissolved organic nitrogen. According to our tests, less than 1% of this would have been released as ammonium during the diffusion treatment. The atom % ^{15}N in the diffused samples was not seriously influenced by this contamination (Table 3). Nor was it significantly affected by the addition of an amount of organically-bound nitrogen, in the form of serine or urea, equalling the amount of ammonium subjected to diffusion. In this experiment, unlabelled compounds were added to a solution of isotopically labelled ammonium (Table 3). In another experiment (not shown), the inclusion of 1 µM N of labelled urea (99 at% ^{15}N) with an equal quantity of unlabelled ammonium did not cause a significant increase in the isotope ratio in the recovered sample.

Fractionation. Fig. 1 suggests some initial fractionation between ^{14}N and ^{15}N during diffusion. However, using the diffusion time recommended (6 h), fractionation should be no problem in calculating the uptake and regeneration rates in this type of experiments.

The atom % ^{15}N decreased slightly after 6 h diffusion (Fig. 1). In similar time course experiment with DDW the atom % ^{15}N was the same after 24 h and 48 h diffusion indicating that ammonium did not diffuse through the wall of the bottles. The decrease in atom % ^{15}N in Fig. 1 was probably caused by ammonium originating from decomposition of dissolved organic nitrogen in the seawater, or, less likely, ammonium dissolving from the bottle itself.

Laboratory test. The diffusion method was tested in a mixture of the rotifer *Brachionus plicatilis* and the prasinophycean alga *Tetraselmis* sp., grown at 20°C. Cultures of the 2 organisms were mixed the day before the ^{15}N experiment. On the day of experimentation, the 10 l culture mixture contained 16.5 ind. ml^{-1} of *B. plicatilis* and 2600 cells ml^{-1} of *Tetraselmis*. The concentration of ammonium, part of which was due to ^{15}N-labelled ammonium chloride added at the start of incubation, was about 4 μM so it was not necessary to add carrier. The culture was sampled initially and after 1.7, 3 and 6 h. The samples were filtered before they were prepared for diffusion. Fig. 3a shows the decrease in the atom % ^{15}N of the ammonium pool during the 6 h incubation time. These data were used together with simultaneous ammonium analyses (Fig. 3a) in a calculation of nitrogen turnover according to Blackburn (1979). This calculation, after correcting for contaminating unlabelled nitrogen, yielded ammonium regeneration rates of 0.41, 0.48 and 0.35 $\mu M\ h^{-1}$ and uptake rates of 0.35, 0.29 and 0.11 $\mu M\ h^{-1}$ after 1.7, 3 and 6 h incubations. Grazing took a heavy toll on the *Tetraselmis* cells during the incubation and is the likely reason for the decline in ammonium uptake rates.

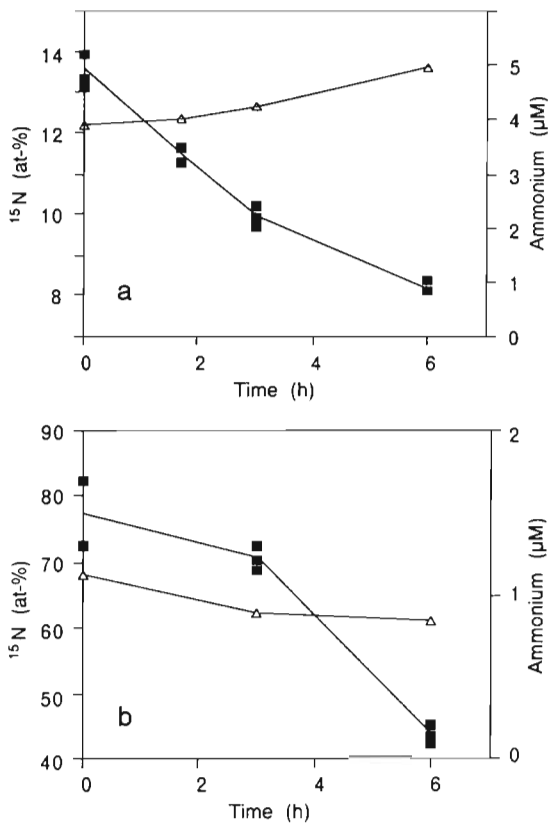

Fig. 3. (a) Regeneration experiment with a mixed culture of *Brachionus plicatilis* and *Tetraselmis* sp., showing changes in atom % ^{15}N (■) and ammonium concentration (△) versus incubation time. (b) Regeneration experiment with a plankton sample from the Oslofjord on 26 May 1988. Symbols as in (a)

Field test. A trial with natural plankton was conducted with a sample from the Oslofjord, Norway, collected on 26 May 1988 and incubated on deck in a 8 l bottle. The plankton consisted of a mixture of the diatom *Skeletonema costatum* and various autotrophic and heterotrophic flagellates, with some metazoan microzooplankton. In this case, highly labelled (95 at % ^{15}N) ammonium chloride was added to give a total ammonium concentration of about 1 μM. Samples were withdrawn initially and after 3 and 6 h incubation. Prior to diffusion, 0.75 µmol of unlabelled ammonium chloride was added as a carrier to each 250 ml sample. The isotope ratios, corrected for carrier N, are shown in Fig. 3b together with the ammonium concentrations. Calculation yielded ammonium regeneration rates of 0.03 and 0.14 $\mu M\ h^{-1}$ and uptake rates of 0.11 and 0.16 $\mu M\ h^{-1}$ after 3 and 6 h incubations.

Potential sources of error. In our experience contamination by nitrogen in reagents, and also by dissolved organic nitrogen in seawater, may cause serious errors in the recently developed extraction methods for determining the isotope content of dissolved ammonium (Dudek et al. 1986, Selmer & Sörensson 1986). With the present method, the sum of nitrogen introduced by reagents and handling and the nitrogen contributed by organic N compounds ordinarily present in seawater appears to be of the order of 0.1 µmol N per sample (corresponding to an ammonium concentration of about 0.5 μM). This is quite acceptable and is easily corrected for in routine work. However, there is a possibility that there could be a larger interference of dissolved organic nitrogen in bloom situations or in heavily enriched water, and this we have not been able to test so far.

One puzzling observation is that there is an apparent increase in ^{15}N content if samples of natural isotope composition (0.37 at% ^{15}N) are subjected to diffusion, even if new bottles are used (Table 2). The only explanation for this that we can find is that the excess sulphuric acid present on the glass-fibre filter strips during diffusion, which afterwards is included with the sample in the discharge tube, interferes with the optics in the emission spectrometry. Sulphuric acid spotted on glass-fibre filter strips together with standards (0.37 at % ^{15}N, 25.19 at % ^{15}N) and put directly into discharge

tubes did not increase the ^{15}N content significantly (values not given). Consequently it is not the acid as such which causes the seeming increase in the ^{15}N content in diffused samples of natural isotope composition. Isotope enrichments close to background are not ordinarily used in ammonium regeneration experiments, and the effect is not noticeable at enrichments of 8 at% ^{15}N or higher (Table 2). However, future users of the method should bear the effect in mind as one possible complication if low enrichment is used.

Perspectives. It is apparent from the growing literature on ammonium regeneration in the marine microplankton (see Selmer 1988 for a review) that a full description of the processes involved requires frequent sampling in time and space. Use of direct diffusion will facilitate this by eliminating some of the more cumbersome steps in the collection of data. We are now making use of this method in ongoing and projected work in the Barents Sea, in the Antarctic, and in Norwegian coastal water.

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LITERATURE CITED

Blackburn, T. H. (1979). Method for measuring rates of NH_4^+ turnover in anoxic marine sediments, using a ^{15}N-NH_4^+ dilution technique. Appl. environ. Microbiol. 37: 760–765

Caperon, J., Schell, D., Hirota, J., Laws, E. (1979). Ammonium excretion in Kaneohe Bay, Hawaii, measured by a ^{15}N isotope dilution technique. Mar. Biol. 54: 33–40

Dudek, N., Brzezinski, M. A., Wheeler, P. A. (1986). Recovery of ammonium nitrogen by solvent extraction for the determination of relative ^{15}N abundance in regeneration experiments. Mar. Chem. 18: 59–70

Fisher, T. R., Morrissey, K. M. (1985). Methods for the recovery of ammonium from natural waters for measurement of ^{15}N composition in isotope dilution experiments. Mar. Chem. 16: 11–21

Glibert, P. M. (1988). Primary productivity and pelagic nitrogen cycling. In: Blackburn, T H., Sørensen, J. (eds.) Nitrogen cycling in coastal marine environments. Wiley, Chichester, p. 3–31

Glibert, P. M., Lipschultz, F., McCarthy, J. J., Altabet, M. (1982). Isotope dilution models of uptake and remineralization of ammonium by marine plankton. Limnol. Oceanogr. 27: 639–650

Kristiansen, S., Paasche, E. (1982). Preparation of ^{15}N-labelled phytoplankton samples for optical emission spectrometry. Limnol. Oceanogr. 27: 373–375

McCarthy, J. J., Carpenter, E. J. (1983). Nitrogen cycling in near-surface waters of the open ocean. In: Carpenter, E. J., Capone, D. G. (eds.) Nitrogen in the marine environment. Academic Press, New York, p. 487–512

Paasche, E. (1988). Pelagic primary production in nearshore waters. In: Blackburn, T. H., Sørensen, J. (eds.) Nitrogen cycling in coastal marine environments. Wiley, Chichester, p. 33–57

Paasche, E., Kristiansen, S. (1982). Ammonium regeneration by microzooplankton in the Oslofjord. Mar. Biol. 69: 55–63

Probyn, T. A. (1987). Ammonium regeneration by microplankton in an upwelling region. Mar. Ecol. Prog. Ser. 37: 53–64

Selmer, J.-S. (1988). Ammonium regeneration in eutrophicated coastal waters of Sweden. Mar. Ecol. Prog. Ser 44: 265–273

Selmer, J.-S., Sörensson, F. (1986). New procedure for extraction of ammonium from natural waters for ^{15}N isotopic ratio determinations. Appl. environ. Microbiol. 52: 577–579

Solórzano, L. (1969). Determination of ammonia in natural waters by the phenolhypochlorite method. Limnol. Oceanogr. 14: 799–801

Solórzano, L., Sharp, J. H. (1980). Determination of total dissolved nitrogen in natural waters. Limnol. Oceanogr. 25: 751–754.

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